

Contents lists available at [ScienceDirect](http://ScienceDirect.com)

Electronic Journal of Biotechnology



A rapid method for an offline glycerol determination during microbial fermentation

Jennifer Kuhn^a, Hagen Müller^b, Denise Salzig^{a,*}, Peter Czermak^{a,b,c,d}^a Institute of Bioprocess Engineering and Pharmaceutical Technology, University of Applied Sciences Mittelhessen, Wiesenstrasse 14, 35390 Giessen, Germany^b Departments of Chemical Engineering, Kansas State University, Manhattan, KS, USA^c Faculties of Biology and Chemistry, University of Giessen, Germany^d Fraunhofer Institute for Molecular Biology and Applied Ecology, Project Group "Bioresources", Winchesterstrasse 3, 35394 Giessen, Germany

ARTICLE INFO

Article history:

Received 27 February 2014

Accepted 30 October 2015

Available online 7 February 2015

Keywords:

Glycerol determination

Fermentation broth

Hantzsch reaction

Malaprade reaction

ABSTRACT

Background: The purpose of this work was to find a rapid method for glycerol detection during microbial fermentations. The method requirements were, first, to avoid sample pretreatment, and second, to measure glycerol precisely especially out of fermentation broth.

Results: This was achieved by combining two reaction principles — the Malaprade reaction and the Hantzsch reaction. In the Malaprade reaction, glycerol is converted into formaldehyde. This forms a dye in the Hantzsch reaction after which adsorption is then detected. The subsequent assay was investigated with two different fermentation media, a chemically undefined and a chemically defined media, used for *Pichia pastoris* fermentation. In both media, as well as in real fermentation samples, glycerol content could be reproducibly detected with the method. Moreover, measurements were more precise than using a standard glycerol detection kit.

Conclusions: With this rapid assay, glycerol could be detected easily in microbial fermentation broth. It is reliable over a wide concentration range including advantages such as an easy assay set-up, a short assay time and no sample pretreatment.

© 2015 Pontificia Universidad Católica de Valparaíso. Production and hosting by Elsevier B.V. All rights reserved.

1. Introduction

For a multitude of bacterial or yeast fermentations, glycerol is a frequently used carbon source. Most standard methods used to determine the glycerol content in fermentation media use the high performance liquid chromatography (HPLC) with a refractive index detector. Due to the number of samples taken during the fermentation and the need of a complex sample preparation, the HPLC measurement is not only time-consuming but also cost-intensive. In addition, several glycerol detection kits are available which are based on enzymatic reactions and spectrometric detection of a dye. Unfortunately these assays are often developed for e.g. food samples, plasma or serum and are not suitable for fermentation samples. Thus the focus in this work was to find a simple and fast glycerol detection method without the need for pretreatment of the fermentation samples. This was achieved by combining the Malaprade reaction and the Hantzsch reaction. This choice was done because methods based on the Malaprade reaction are the ones that are most widely used for the detection of 1,2-diols or related compounds including glycerol. In

this reaction, glycerol is converted with periodate into formaldehyde (Fig. 1) [1].

Afterwards, the Hantzsch reaction is used for the detection of formaldehyde. In this reaction, acetylacetone and ammonia are used to form a dye in connection with formaldehyde (Fig. 2). This dye is 3,5-diacetyl-1,4-dihydrolutidine (DDL), which can be measured at 410 nm in a spectrophotometer [2].

The assay can be done in a standard spectrophotometer or a microplate reader. Using the microplate reader has the advantage that more samples (e.g. 96 or 384, dependent on the well plate) can be measured at once.

2. Materials and methods

2.1. Fermentation media

The chemically undefined medium BMGY contained the following ingredients: 10 g L⁻¹ yeast extract, 20 g L⁻¹ peptone, 11.9 g L⁻¹ KH₂PO₄ and 2.1 g L⁻¹ K₂HPO₄ (pH 6.0) dissolved in water. pH adjustment was done with 1 M NaOH if needed. After autoclaving (121°C, 20 min), sterile-filtrated yeast nitrogen base (final concentration 13 g L⁻¹) and sterile-filtrated biotin (final concentration 4 g L⁻¹) were added to the solution [3].

* Corresponding author.

E-mail address: denise.salzig@kmub.thm.de (D. Salzig).

Peer review under responsibility of Pontificia Universidad Católica de Valparaíso.

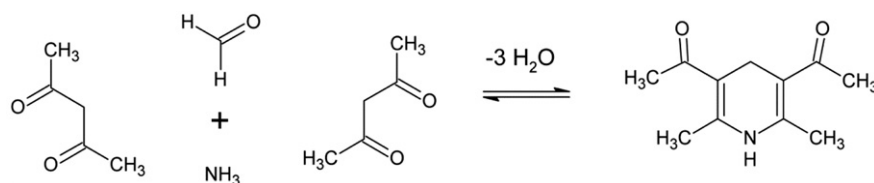


Fig. 1. Reaction scheme of the Malaprade reaction. In this reaction, sodium periodate is used to convert glycerol into formaldehyde.

The chemically defined medium FM22 consisted of 42.9 g L⁻¹ KH₂PO₄, 5.0 g L⁻¹ (NH₄)₂SO₄, 1.0 g L⁻¹ CaSO₄·2H₂O, 14.3 g L⁻¹ K₂SO₄, 5.2 g L⁻¹ Na₃C₆H₅O₇ and 11.7 g L⁻¹ MgSO₄·7H₂O (pH 5.0). pH adjustment was done with ammonia solution (30%, Carl Roth) [4]. The medium was autoclaved at 121°C for 20 min. Additionally 8.7 mL L⁻¹ of the sterile-filtrated trace-metal solution was added. The trace-metal solution (PTM1) contained 6.0 g L⁻¹ CuSO₄·5H₂O, 0.08 g L⁻¹ NaI, 3.0 g L⁻¹ MnSO₄·H₂O, 0.2 g L⁻¹ Na₂MoO₄·2H₂O, 0.02 g L⁻¹ H₃BO₃, 0.5 g L⁻¹ CoCl₂, 20 g L⁻¹ ZnCl₂, 65 g L⁻¹ FeSO₄·7H₂O, 0.2 g L⁻¹ biotin and 5.0 mL L⁻¹ H₂SO₄ [4].

Autoclaved glycerol was added to both media in different concentrations for assay validation. During the fermentation, the initial glycerol content in the medium was 15 g L⁻¹.

2.2. Fermentation of *Pichia pastoris*

For the fermentation, the *Pichia* Pink Expression System from Invitrogen was used [3]. A 50 mL overnight-culture was done in shaking flasks. 7 µL antifoam (Struktol J673A, Schill + Seilacher) was added to the medium. Inoculation of the pre-culture was done with 1 mL of a cryo-culture (OD₆₀₀ ~ 5). The pre-culture was grown at 30°C and 250 rpm for approx. 16 h. The fermentation was performed in a 20 L stirred tank reactor (20 L Bench Top Reactor, Applikon, Schiedam, Netherlands). Working volume was 7 L. The fermentation medium was prepared as described above. Before inoculation 1 mL sterile antifoam was added. The fermentation parameters were set to 30°C, 800 rpm and an aeration rate of 1 vvm. The medium was inoculated with the pre-culture to a final OD₆₀₀ of 0.1. Cells were grown for 24 h.

2.3. Sampling

For the detection of glycerol and biomass, samples were taken from the reactor. Therefore every h 1 mL fermentation broth was taken out of the vessel. 500 µL was immediately used for biomass determination. The other 500 µL was centrifuged (5 min, 5000 × g). The supernatant was transferred into a new tube and stored at -20°C for glycerol determination.

2.4. Biomass determination

The growth of the culture was controlled by measuring the optical density at 600 nm (OD₆₀₀) of the fermentation broth with a standard spectrophotometer. Samples were diluted with fermentation medium until OD₆₀₀ reaches values between 0.1 and 1. With a correlation between the optical density and the biomass dry weight, the biomass content was calculated (CDW in g L⁻¹ = 0.47 · OD₆₀₀). Therefore a concentration series of *P. pastoris* biomass was prepared. Each sample

(15 mL) of the concentration series was both measured at OD₆₀₀ and for CDW (cell dry weight). For CDW determination 10 mL of the sample was centrifuged, washed with ddH₂O, centrifuged again and resuspended in ddH₂O. The solution was transferred to a weighted beaker and dried at 100°C until constant weight.

2.5. Glycerol detection combining the Malaprade reaction and the Hantzsch reaction

For the assay, two reagents were needed. Reagent I, the periodate reagent, consisted of 18 mg mL⁻¹ sodium periodate (Merck) dissolved in distilled water with 10% (v/v) acetic acid (Merck). For preparation purposes, sodium periodate was first dissolved in water. After the addition of acetic acid and adequate mixing, 77 mg mL⁻¹ ammonium acetate (VWR) was added. The amount of sodium periodate in this reagent was calculated for a calibration curve from 50 mg L⁻¹–200 mg L⁻¹ glycerol. Reagent II, the acetylacetone reagent, was composed of 1% (v/v) acetylacetone (VWR) in isopropyl alcohol (Roth). This reagent had to be stored in the dark.

The measurement was executed in a microplate reader (Synergy HT BioTek, Winooski, USA). Therefore, an amount of 40 µL sample (cell-free supernatant of the fermentation) was pipetted into each well of a standard 96 well plate (Greiner). Then, 40 µL Reagent I was added and mixed adequately. After an incubation time of 10 min, 125 µL Reagent II was pipetted into each well and mixed adequately. The absorption at 410 nm was measured over a period of 25 min in the plate reader. The glycerol content was calculated with Eq. (1), based on a glycerol standard curve (50 mg L⁻¹–200 mg L⁻¹).

$$\text{glycerol content} \left[\frac{\text{mg}}{\text{L}} \right] = \frac{\Delta A_{\text{sample}(25 \text{ min}-15 \text{ min})} - \Delta A_{\text{blank}(25 \text{ min}-15 \text{ min})}}{\text{slope of the calibration curve} \left[\frac{1}{\text{mg}} \right]} \quad [\text{Equation 1}]$$

The assay can also be performed in a cuvette using a spectrophotometer. In this instance, 5-fold the amount of sample and reagents must be used.

2.6. Measurement of glycerol using a standard assay

The assay was performed in a microplate reader according to the microplate assay procedure in the Glycerol GK Kit manual from Megazyme [5]. In this assay, the glycerol is phosphorylated with the use of adenosine-5'-triphosphate (ATP). The formed adenosine-5'-diphosphate (ADP) is used for the phosphorylation of D-glucose which is oxidized with the formation of nicotinamide-adenine

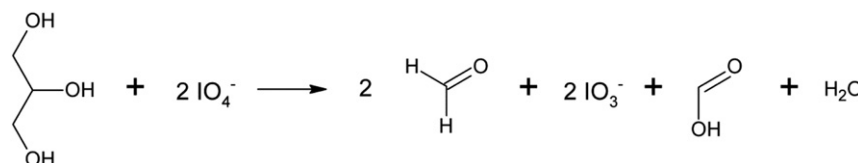


Fig. 2. Reaction scheme of the Hantzsch reaction. In this reaction, formaldehyde is converted into DDL with ammonia and acetylacetone. This dye can be measured at 410 nm.

dinucleotide (NADH). The amount of NADH is detected by a measurement in a spectrophotometer at 340 nm.

2.7. Statistical analysis

For all quantitative data, mean (\bar{x}) and standard deviation (s) were calculated ($n = 3$). The confidence intervals were calculated with a confidence level of 95% and the highest standard deviations of the measured values in percentage ($s\%$). For the validation of the modified assay with samples of known glycerol concentrations, a standard deviation of 6.6% was used for the calculation of the confidence interval. In the measurement done with the Glycerol GK Kit, the confidence interval was calculated with a standard deviation of 11.5%. The confidence interval (CI) was calculated as follows:

$$CI = \bar{x} \pm t_{1-\alpha, n-1} \frac{s\% \bar{x}}{\sqrt{n}} \frac{100\%}{100\%}$$

for $(n - 1) = 2$ and a confidence interval of 95% t has a value of 4.303.

3. Results

3.1. Validation of the modified glycerol assay

For the validation of the modified glycerol assay, the glycerol content in two different yeast fermentation media, a chemically undefined (BMGY) and a chemically defined medium (FM22), was analyzed. To do this, medium samples with known glycerol concentrations from 50 mg L⁻¹ to 200 mg L⁻¹ were prepared and measured.

In Fig. 3, the desired glycerol concentrations in the samples were plotted against the concentrations measured with the modified assay. It was shown that in lower glycerol concentrations, the assay is more accurate than in higher concentrations. Nonetheless, all measured values are inside the confidence interval with a confidence level of 95%. This showed that the assay produced significant values.

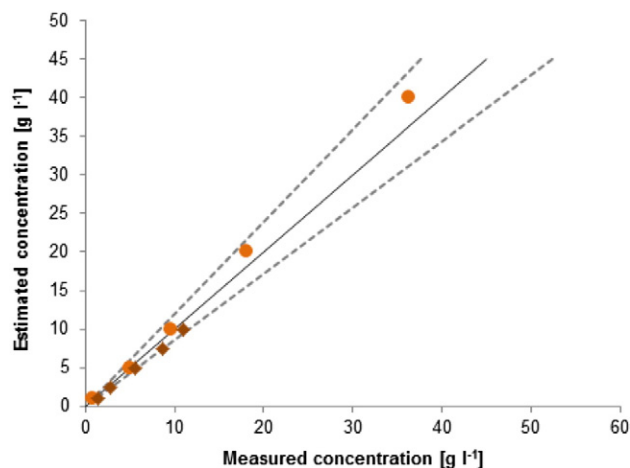


Fig. 3. Validation of the assay with defined samples of glycerol in BMGY (diamonds) and FM22 medium (circles). The samples were measured as triplicates. The standard deviation of the values was between 1.8 and 6.6%. With the highest standard deviation of 6.6%, the confidence interval (dotted line) (with a confidence level of 95%) was calculated. The solid line describes the ideal progression in which the measured values correspond to the desired values. The measurements were performed according to materials and methods.

3.2. Comparison of the modified glycerol assay with a standard glycerol detection kit

After the validation, the modified glycerol assay was compared with a standard glycerol detection method. Therefore, the Glycerol GK Kit from Megazyme was used. The samples with known glycerol concentrations were prepared in the same way than the samples in the validation were.

In Fig. 4, the results of the measurement with the Glycerol GK Kit are shown. The BMGY medium samples, as well as the FM22 medium samples, had a high standard deviation up to 11.5%. Compared to the modified assay, this is a 2-fold increase. Moreover, using the Glycerol GK Kit in low glycerol concentrations, the values are outside of the 95% confidence interval. In consequence, low glycerol concentrations in fermentation broth are not reliably detectable with the Glycerol GK Kit. With regard to the results from the validation and the comparison with the Glycerol GK Kit, the modified glycerol assay seemed to be more suitable for the measurement of glycerol in yeast fermentation medium. The assay avoids sample pretreatment and can be used for glycerol determination in a broad concentration range.

3.3. Glycerol determination of unknown fermentation samples

So far, the new assay has been validated only with pure fermentation media with known glycerol concentrations. As in real fermentations, supernatant is used; the assay was performed with fermentation samples from a *P. pastoris* fermentation in FM22 medium. The glycerol content of the samples was unknown except the sample from the fermentation start. It should be investigated if components of the fermentation supernatant interfere with the assay or if the results stayed unaffected. FM22 has a starting glycerol concentration of 15.0 g L⁻¹. With the modified glycerol assay, a glycerol concentration of 15.5 g L⁻¹ was determined at the fermentation start. This means that the desired and the measured values correlated very well in fermentation supernatants. Therefore, the assay seemed to be very robust against interfering substances as e.g. yeast proteins from the fermentation supernatant. As the assay was always performed at room temperature and as the samples always had a defined pH (pH 6 in BMGY and pH 5 in FM22) the impact of temperature and pH on test stability was not investigated. Components during fermentation which could interfere with the test are produced metabolites or increasing

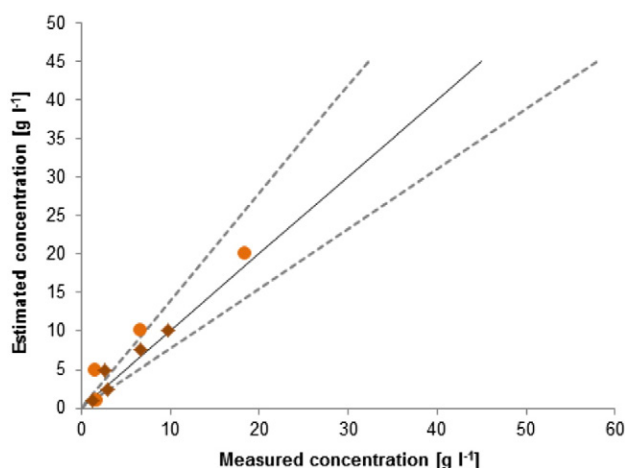


Fig. 4. Measurement of defined samples of glycerol in BMGY (diamonds) and FM22 medium (circles) with the Glycerol GK Kit from Megazyme. The samples were measured as triplicates. The standard deviation of the values was between 1.1 and 11.5%. With the highest standard deviation of 11.5%, the confidence interval (dotted line) with a confidence level of 95% was calculated. The solid line describes the ideal progression in which the measured values correspond to the desired values. The measurements were performed according to materials and methods.

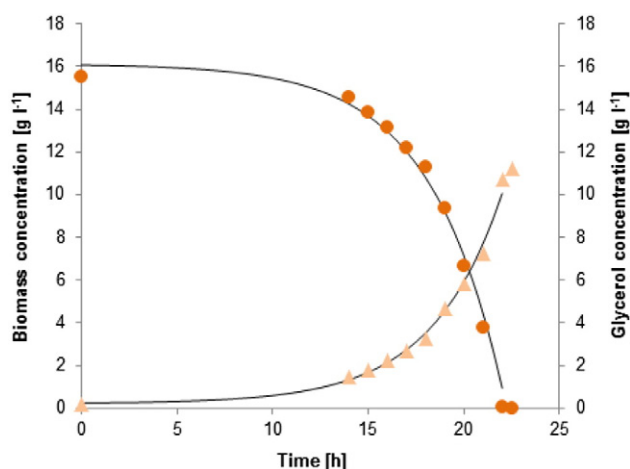


Fig. 5. Measurement of glycerol samples in a fermentation of *P. pastoris* in FM22 medium. The measurements were performed in triples according to the [Materials and methods](#) section. The standard deviation of the glycerol samples (circles) was between 1.9 and 4.4% and the standard deviation of the biomass samples (triangles) was between 0.7 and 1.6%. The solid lines show a regression curve for the glycerol concentration and the biomass concentration.

salt concentrations (mainly Na^+ , OH^- and NH_4^+) due to pH regulation during fermentation. Another study intensely investigated the influence of some salts and medium compounds on a similar glycerol assay. They showed that methanol, ethanol and propanol (5 g L^{-1}), acetic, tartaric and oxalic acids (each 1 g L^{-1}) and sodium pyruvate (1.25 g L^{-1}) had no influence on the test outcome. Furthermore, Na^+ (100 mmol L^{-1}) did not significantly interfere with their test [6]. In our experiments we determined reliable glycerol values independent from the fermentation medium or the fermentation time. Therefore it can be assumed that medium changes during fermentation should not interfere with the test reactions.

The results of the glycerol measurements of the fermentation samples are shown in [Fig. 5](#). The samples showed the expected glycerol decrease during the growth of the culture. With the measured values, a specific maximum glycerol consumption rate of $0.36 \text{ g g}^{-1} \text{ h}^{-1}$ was calculated. This correlates with the glycerol consumption rate of $0.37 \text{ g g}^{-1} \text{ h}^{-1}$ for a fermentation of *P. pastoris* in the same chemically defined FM22 medium [7]. According to this, the assay was not only suitable for the detection of glycerol in pure fermentation medium,

but also for the detection in real fermentation samples. Although the assay was only tested in fermentations with FM22 medium, it can be assumed that the assay can also be used for the detection of glycerol in other media during fermentation. So far, the assay has also been investigated for glycerol determination during *Escherichia coli* fermentations, which has given reliable results.

In conclusion, the modified glycerol assay, based on the Malaprade reaction and the Hantzsch reaction, reliably determines glycerol during microbial and yeast fermentations. It combines several advantages including an easy assay set-up, a short assay time and no sample pretreatment. The assay is applicable over a wide concentration range. The microplate format of the assay allows a high sample throughput. This enables the fast and reliable measurement of many fermentation samples.

Financial support

The authors would like to thank the Hessen State Ministry of Higher Education, Research and the Arts for the financial support within the Hessen initiative for supporting scientific and economic excellence (LOEWE program).

References

- [1] Verma KK, Gupta D, Sanghi SK, Jain A. Spectrophotometric determination of periodate with amodiaquine dihydrochloride and its application to the indirect determination of some organic compounds via the Malaprade reaction. *Analyst* 1987;112: 1519–22. <http://dx.doi.org/10.1039/AN9871201519>.
- [2] Salthammer T. Photophysical properties of 3,5-diacetyl-1,4-dihydroxylutidine in solution: Application to the analysis of formaldehyde. *J Photochem Photobiol A* 1993; 74:195–201. [http://dx.doi.org/10.1016/1010-6030\(93\)80117-R](http://dx.doi.org/10.1016/1010-6030(93)80117-R).
- [3] Invitrogen. PichiaPink™ Expression System [User guide]. [cited January 26, 2015]. Available from Internet at: https://tools.lifetechnologies.com/content/sfs/manuals/pichiapink_expression_system_man.pdf; 2014.
- [4] Jacobs PP, Inan M, Festjens N, Hausteraete J, Van Hecke A, Contreras R, et al. Fed-batch fermentation of GM-CSF-producing glycoengineered *Pichia pastoris* under controlled specific growth rate. *Microb Cell Fact* 2010;9:93. <http://dx.doi.org/10.1186/1475-2859-9-93>.
- [5] Megazyme. Glycerol GK assay procedure. [Manual an auto-analyser; rapid and simple glucokinase format]. [cited January 26, 2015]. Available from Internet at: http://secure.megazyme.com/files/BOOKLET/K-GCROLGK_1212_DATA.pdf; 2012.
- [6] Horstkotte B, Werner E, Seresht AK, Cornelissen G, Elsholz O, Cerdà Martín V, et al. Sequential injection analyzer for glycerol monitoring in yeast cultivation medium. *Talanta* 2007;71:941–7. <http://dx.doi.org/10.1016/j.talanta.2006.05.072>.
- [7] Jahic M, Rotticci-Mulder J, Martinelle M, Hult K, Enfors SO. Modeling of growth and energy metabolism of *Pichia pastoris* producing a fusion protein. *Bioprocess Biosyst Eng* 2002;24:385–93. <http://dx.doi.org/10.1007/s00449-001-0274-5>.